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Immunogenicity Studies with Haptenated Melittin Peptides: Implication for Membrane Involvement during the Recognition Step

VÉRONIQUE CURCIO-VONLANTHEN, HANSPETER ROLLI and CONRAD H. SCHNEIDER

University of Bern, Institute of Immunology and Allergology, Inselspital, Bern Switzerland

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Abstract: Melittin peptides carrying 2,4-dinitro-6-carboxyphenyl (Dncp) haptenic groups regularly evoked anti-hapten IgG responses in mice or guinea pigs when the hapten was C-terminally attached. Single haptens on the N-terminal helix in several positions gave poor or no responses in the early stages but adequate titres after prolonged immunization. Peptides with Dncp at the C-terminus as an invariant feature and a second Dncp in various positions along the peptide chain did not fail to produce adequate responses. The hampering effect is not due to a defect at the T-cell level but involves the recognition step on the B-cell. It is implied that the haptenic interaction with the paratope of the recognizing immunoglobulin on the B-cell involves the cell membrane in an important way. It is also suggested that late antibody responses should not be overlooked during the development of proteinaceous immunogens for vaccination.

Keywords: melittin immunogenicity; antigen recognition; membrane involvement; haptenic position; late IgG responses; cellular dynamics

INTRODUCTION

Although the relatively high immunogenicity of melittin and melittin-derived peptides observed in several species is not entirely understood [1] this property is certainly an asset and may be used in the study and further elucidation of antibody-generating processes. Melittin possesses a remarkably strong B-cell epitope at its highly basic C-terminal end as demonstrated in rabbit [2] as well as murine immunizations [3, 4]. However, in rabbits, the N-terminal chain and the middle segment serve as epitopes as well which means that there is almost a continuum of B-cell epitopes in this comparably small peptide immunogen [2]. Of interest is the conclusion in the same report that the antibodies generated against the N-terminal moiety are in fact directed against the helical and not the linear form of the chain.

Melittin peptides have also been used as haptenic carriers [1]. Good anti-haptenic IgG responses in guinea pigs were regularly obtained when a single Dncp-haptenic group was attached to the C-terminal end but we found poor responses with the same hapten at the N-terminus. A similar observation was made by King *et al.* [5, 6] who reported that a melittin analogue with a lactosyl group at its C-terminus gave good lactoside-specific murine antibody responses whereas a conjugate with N-terminal lactoside failed to produce anti-lactoside antibodies. This result excludes the possibility that the observations with Dncp are related to this hapten only and shows that such data may be obtainable with a variety of haptenic groups. We tentatively concluded that the hampering effect is not related to functions at the T-cell level because N-terminal substituents such as lauryl or caprylyl did not affect the anti-Dncp-responses of C-terminally monohaptenated peptides. Furthermore the anti-Dncp response of melittin

Abbreviations: Dncp, 2,4-dinitro-6-carboxyphenyl; PBS, phosphate-buffered saline, 0.01 M, pH 7.4.

Address for correspondence: Prof. C.H. Schneider, PO Box 10, Inselspital CH-3010 Bern, Switzerland.

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peptides with Dncp-groups at both ends was not impaired [1].

In this paper we show that anti-peptidic in contrast to haptenic responses in N-terminally haptenated peptides are not impaired and therefore confirm that T-cell function is not impaired. We further show that the hampering effect also occurs with haptens in other positions and that dynamic aspects have to be involved because the poor anti-haptenic responses do not remain so upon prolonged immunization. The data imply that the B-cell membrane is involved during interaction of the peptide conjugates with the membrane-bound specific immunoglobulins engaged in the recognition step.

MATERIALS AND METHODS

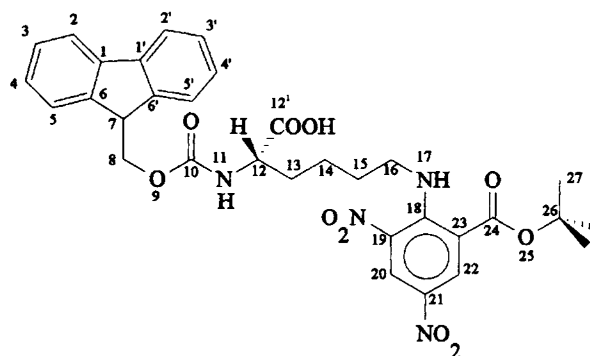
Synthetic Peptides

Solid-phase peptide synthesis according to the Fmoc/tert-butyl strategy followed essentially the lines described previously [1]. The Dncp haptenic group was introduced in all conjugates by using Fmoc-Lys(*t*-butyl-Dncp)-OH (structure A) as a building block. DMF was the reaction solvent and a *p*-alkoxybenzylalcohol resin carrying Fmoc-Gly as the first C-terminal amino acid was used in all preparations. Resin and amino acid derivatives were obtained from Bachem, Bubendorf (Switzerland). Couplings were done with TBTU in the presence of diisopropylethylamine. Cleavage of the conjugate from the resin was achieved by treatment of the resin (0.2–1 g) overnight with 2.5 ml thioanisol, 2.5 ml H_2O, 2.5 g phenol and 40 ml TFA. Purification was performed by Sephadex G-25 gel filtration and on a preparative Nucleosil C-18 HPLC reversed-phase column. The final products were characterized by amino acid analysis and electrospray mass spectrometry (Table 1).

Fmoc-Lys(*t*-butyl-Dncp)-OH (structure A)

To 4.3 mmol Fmoc-Lys-OH in 15 ml CH_2Cl_2 triethylamine was added until the pH reached 9.0, followed by 1.3 g (4.3 mmol) *t*-butyl-Dncp-Cl [7] in CH_2Cl_2. After 4 h stirring at ambient temperature the solvent was evaporated and small volumes of CH_2Cl_2 were added four times and evaporated each time. The residual oil was taken up in $CHCl_3$/MeOH, 9 : 1, and chromatographed with this solvent on a 150 g silica gel 60 column (5 × 21 cm, particle size 0.063–0.2 mm, Merck, Darmstadt). The product peak was identified by TLC and the relevant fractions were pooled, evaporated and lyophilized after addition of H_2O. Yield: 1.97 g, 72%. TLC ($CHCl_3$: MeOH, 9 : 1) R_F 0.29, homogeneous; TLC (dioxane : H_2O, 9 : 1) R_F 0.88, homogeneous. Detection of spots on the silica gel plates (F 254, 0.25 mm, Merck, Darmstadt) was by UV at 354 nm and visual inspection. ^{1}H-NMR (360 MHz, $CDCl_3$): δ 9.42 (s br, 1H, $HO_2C(12^1)$); 8.80 (d, J = 2.7 Hz, 1H, H–C(20)); 8.76 (d, J = 2.5 Hz, 1H, H–C(22)); 7.75 (d, J = 7.5 Hz, 2H, H–C(2, 2′); 7.58 (d, J = 7.4 Hz, 2H, H–C(5, 5′); 7.39 (t, J = 7.4 Hz, H–C(4, 4′); 7.30 (td, J = 7.4 Hz, J = 1.1 Hz, 2H, H–C(3, 3′); 5.31 (d, J = 8.2 Hz, HN(11, 17); 4.41 (t, J = 6.8 Hz, 2H, H_2–C(8)); 4.21 (t, J = 6.9 Hz, 1H, H–C(7)); 2.0–1.2 (m, 8H, H_2–C(13, 14, 15, 16)); 1.6 (s, 9H, $H_3C(27)$). H–C(12) can not be seen in the spectrum because of an extremely large signal resulting from a chemical exchange due to a hindered rotation between C(10) and N(11). ^{13}C-NMR (200 MHz, $CDCl_3$): δ 175.7 (C(12^1)); 165.7 (C(24)); 156.1 (C(10)), 148.3 (C(18)); 143.8 + 143.7 (C(6, 6′)); 141.4 (2C, C(1, 1′)); 135.4 (C(21)); 134.2 (C(19)), 131.2 (C(22), 127.8 + 127.1 + 124.9 + 120.0 (8C, C(2, 2′, 3, 3′, 4, 4′, 5, 5′)); 126.8 (C(20)); 116.8 (C(23)), 84.4 (C(26)); 67.2 (C(8)); 53.5 (C(12)); 47.3 (C(7)); 46.3 (C(16)); 31.9 (C(15)); 29.3 (C(13)); 28.14 (C(27)); 22.5 (C(14)).

Dncp-HSA is a human serum albumin conjugate with 14 Dncp groups per molecule as used before [1].



Structure A

ELISA

Polystyrene microtitre plates (Dynatech) were coated with N^1-Dncp- diaminohexane and used as described before [1, 8]. The detecting antibodies, goat anti-guinea pig IgG (H + L) alkaline phosphatase and goat anti-mouse IgG (H + L) alkaline phosphatase, were obtained from Jackson Immunoresearch Laboratories, West Grove, PA. Titres are expressed as the highest reciprocal dilution (*D*) of the antisera giving

Table 1 Synthetic Peptides Derived from Melittin[a]

Melittin[a] peptides, **abbreviations** sequence			Molar ratios of amino acids												MS analysis
			Glu	Ser	Gly	Arg	Thr	Ala	Pro	Val	Ile	Leu	Phe	Lys	
Melittin*(1–26)K(ac)G-(3-K(Dncp)),	**M-3D**	Theoretical	2	1	3	2	2	2	1	2	3	4	1	4	3316.71
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.12	0.86	3.03	2.09	1.95	2.13	1	1.87	2.86	3.94	1.05	4.1	3316.22 ± 0.40
Melittin*(1–26)K(ac)G-(7-K(Dncp)),	**M-7D**	Theoretical	2	1	4	2	2	2	1	2	3	4	1	3	3245.59
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.11	0.86	3.94	2.04	1.92	2.05	1	2	2.97	3.97	1.04	3.08	3244.98 ± 0.33
Melittin*(1–26)K(ac)G-(11-K(Dncp)),	**M-11D**	Theoretical	2	1	4	2	1	2	1	2	3	4	1	4	3272.72
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.15	0.87	3.98	2.06	0.93	2.01	1	1.96	2.94	3.97	1.04	4.09	3272.18 ± 0.51
Melittin*(1–26)K(ac)G-(15-K(Dncp)),	**M-15D**	Theoretical	2	1	4	2	2	1	1	2	3	4	1	4	3302.91
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.07	0.85	3.91	2.06	1.84	1.02	1	2	3	4.06	1.05	4.14	3302.18 ± 0.33
Melittin*(1–26)K(ac)G-(19-K(Dncp)),	**M-19D**	Theoretical	2	1	4	2	2	2	1	2	3	4	0	4	3226.81
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.05	0.88	3.99	2.28	1.86	2	1.06	2	2.91	3.92	0	4.02	3226.12 ± 0.22
Melittin*(1–26)K(ac)G-(23-K(Dncp)),	**M-23D**	Theoretical	2	1	4	2	2	2	1	2	3	4	1	3	3245.81
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.12	1.01	3.94	2.39	1.93	2.02	1.1	1.98	2.84	3.66	1.05	2.96	3245.29 ± 0.17
Melittin(1–26)K(Dncp)G-(3-K(Dncp)),	**MD-3D**	Theoretical	2	1	3	2	2	2	1	2	3	4	1	3	3485.00
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.48	1.1	3.66	2.66	2.27	1.93	1.24	1.02	2.46	2.26	1.13	2.7	3484.17 ± 0.14
Melittin(1–26)K(Dncp)G-(7-K(Dncp)),	**MD-7D**	Theoretical	2	1	4	2	2	2	1	2	3	4	1	2	3413.88
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.07	0.89	4.05	2.3	1.91	1.99	1.09	1.73	2.95	3.78	1.02	2.22	3413.08 ± 0.12
Melittin(1–26)K(Dncp)G-(11-K(Dncp)),	**MD-11D**	Theoretical	2	1	4	2	1	2	1	2	3	4	1	3	3440.94
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.19	0.99	4.22	2.34	0.99	2.05	1.06	1.68	2.77	3.73	1.03	2.97	3440.46 ± 0.38
Melittin(1–26)K(Dncp)G-(15-K(Dncp)),	**MD-15D**	Theoretical	2	1	4	2	2	1	1	2	3	4	1	3	3470.97
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.04	0.89	4.01	2.21	1.88	1.07	1.05	1.97	2.88	3.9	0.99	3.11	3470.97
Melittin(1–26)K(Dncp)G-(19-K(Dncp)),	**MD-19D**	Theoretical	2	1	4	2	2	2	1	2	3	4	0	3	3394.87
GIGAVLKVLTTGLPALISFIKRKRQQKG		*Found*	2.05	0.87	3.92	2.26	1.82	2	1.07	2.01	2.89	3.84	0	3.27	3394.91 ± 0.51

[a] Melittin is a modified sequence containing Phe instead of Trp at position 19 of the original sequence. In melittin*, Lys at position 27 is acetylated. **MD** (melittin(1–26)K(Dncp)G), **DMD** (Dncp–melittin(1–26)K(Dncp)G), and **DM** (Dncp–melittin(1–26)) were described and characterized in [1].

an absorbance of 1.0 after 30 min incubation with 4-nitrophenylphosphate (1 mg/ml in 0.05 M carbonate buffer – 1 mM $MgCl_2$, pH 9.8).

Immunodot Assay

Peptides (5 mg/ml) were applied in small dots (1 µl of peptide in TRIS-buffered saline, 0.05 M, pH 7.6) to nitrocellulose strips which are reacted with the antiserum to be assessed and finally incubated with the same detecting antibody conjugates as in ELISA. The strips were developed with Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate (Sigma, Switzerland) as substrate. The procedure has been described in detail [9]. The results are expressed as relative optical density measured densitometrically on the spot and for reference on a spot treated with TRIS-buffered saline on the same strip.

It should be noted that the Dncp-haptenated melittin peptides but not non-haptenated peptides are capable of binding to the nitrocellulose, showing that Dncp has some special attachment function which may reduce its accessibility. In line with this is the observation generally made, that anti-Dncp antibodies in contrast to anti-peptidic antibodies are only poorly measured in most anti-Dncp-melittin peptide antisera. In other words the immunodot assay measures essentially anti-peptidic and not anti-Dncp titres.

Immunization of animals

GOHI-guinea pigs (out-bred, female, 250 g) were obtained from BRL Ltd, Füllinsdorf, Switzerland, and immunized with Dncp-peptides as described previously [1]. Balb/c mice (female, 3–12 weeks old) were purchased from IFFA, Credo, Saint-Germain sur l'Arbresle, France, and immunized with assistance of the adjuvant TiterMax of CytRx, Norcross, Georgia (USA). The adjuvant essentially contains the block copolymer CRL 89-41, squalene as a metabolizable oil and a microparticle stabilizer. The peptides (2 nmol in 20 µl PBS) were emulsified with 20 µl adjuvant and injected subcutaneously at the base of the tail. Booster injections contained 2 nmol of peptide in 0.1 ml PBS and were given intraperitoneally. Antisera were collected one week after a boost; however, not all animals received frequent boosts. In these cases several weeks may have elapsed between boost and bleedings as can be seen in the text. Antisera were stored in 20 µl portions in the deep-freeze until used.

RESULTS

Characterization of the Immunodot Assays

Antisera against melittin conjugates carrying Dncp at either end or at both ends were tested with the same conjugates on the nitrocellulose strips. The series also included Dncp-HSA as a reference. It is evident from Figure 1 that the highest responses are obtained with the homologous antigens on the strip whereas interactions depending on Dncp as the common feature are weaker or poor such as the interaction of the anti-Dncp-HSA antiserum with dotted **DM**.

Characterization of Murine Anti-Dncp IgG Responses Towards Dncp Terminally Attached to Melittin Peptides

Immunization of Balb/c mice with **MD**, **DMD** and **DM** essentially confirmed the observations [1] previously made in guinea pig immunizations. The ELISA data summarized in Figure 2 show the regular responses with **MD** and **DMD** and the lack of early responses with **DM**. This lack of significant response did not persist in most animals however, and 8 or 11 weeks after priming the titres became comparable to those obtained with **MD** or **DMD**. It can also been seen that the anti-Dncp titres are comparable to those from the haptenated protein Dncp-HSA.

It should be noted that animals in this series received only one boost after ten weeks which means that boosting is not absolutely necessary for the development of the late responses with **DM**. This is

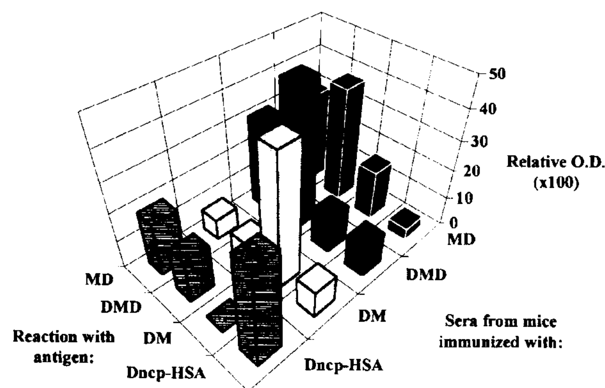


Figure 1 Immunodot assay. Relative O.D. as quantitative measurement for the amount of anti-peptidic and anti-Dncp response in mice immunized with **MD**, **DMD**, **DM** and **Dncp-HSA**. The bars represent mean values from four or five antisera collected six weeks after priming.

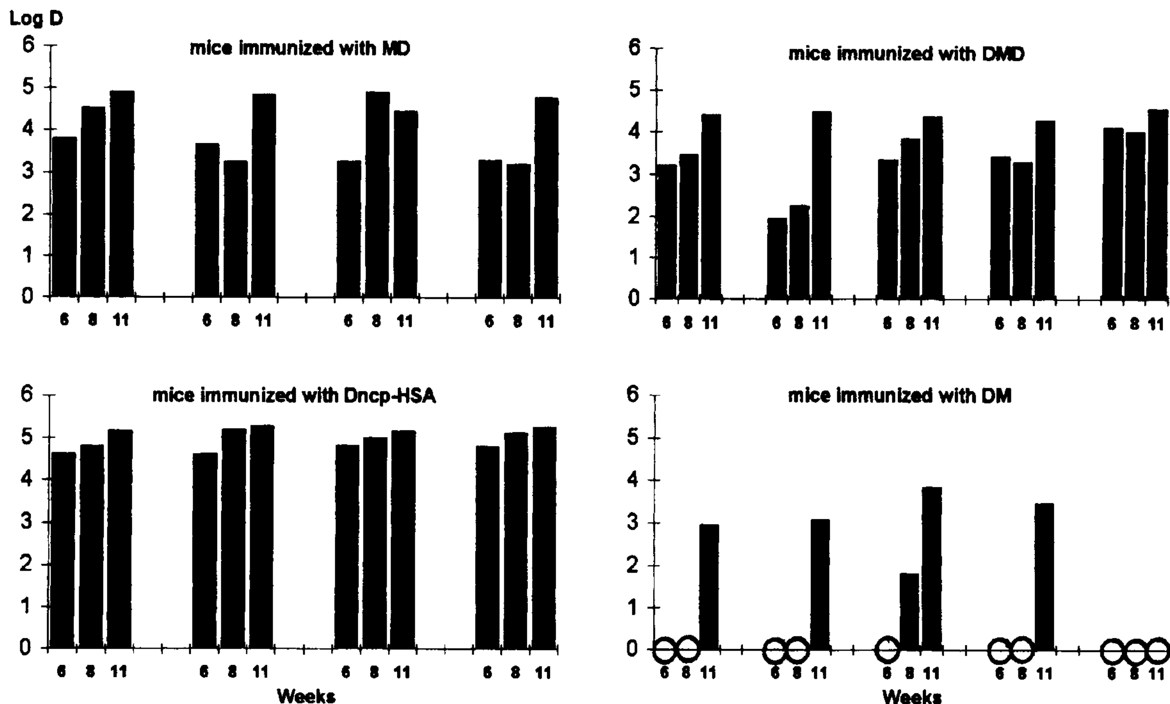


Figure 2 ELISA anti-Dncp titres of mice, after immunization with **MD**, **DMD**, **DM** and **Dncp-HSA**. Each group of columns represents the response of a single animal. The circles mean that the serum titres are ≤ 2.

clearly shown by the third animal in the group immunized with **DM** as it began to show a measurable titre before the boost, namely in the eighth week.

Antibodies against Melittin Peptides with Dncp in Various Positions

The peptide conjugates used as immunogens in murine immunizations in this series were synthesized by replacing positions 3 (Gly), 7 (Val), 11 (Thr), 15 (Ala), 19 (Phe, Trp) and 23 (Lys) by Lys(Dncp) as listed in Table 1. The ELISA data are summarized in Figure 3. It is evident that **M-3D** and **M-7D** fail to immunize during the early stages but produce good responses later, comparable to the results of **DM** of Figure 2. The results of **M-7D** are particularly pronounced since responses are seen only after 11 weeks while two of five animals remained negative. Starting with **M-11D** and up to **M-23D** the responses became good with some late responding animals showing good titres only after six weeks. THe **M-3D**, **M-7D** and **M-11D** primed mice received a single boost after ten weeks. Again as with **DM** of Figure 2 some **M-3D** primed animals began to develop good titres before the boost. The **M-15D**, **M-19D** and **M-23D** primed mice received boosts after five and nine weeks.

Selected antisera from this series were assessed in the immunodot assay. Figure 4 shows the titres obtained from homologous interactions of antisera eight weeks (**M-3D**, **M-7D**, **M-11D**) and six weeks (**M-15D**, **M-19D**, **M-23D**) after priming with the immunizing peptides dotted on the strips. Well-measurable titres are obtained in all cases although the ELISA titres at that stage were still negative. This result confirms that the development of anti-Dncp titres, but not at the same time the development of anti-peptidic titres, is impaired which implies the non-involvement of an impairment at the T-cell level.

Immunodot assays of selected guinea pig antisera gave a similar picture. Relatively good titres were seen with anti-DM antisera at a stage where poor anti-Dncp responses could be measured in ELISA (data not shown).

Anti-Dncp IgG Responses Against Di-haptenated Melittin Peptides

In a control series melittin peptides with Dncp in positions 3, 7, 11, 15 and 19 containing a second C-terminal Dncp (Table 1) were subjected to murine immunogenicity testing. Figure 5 shows that good anti-Dncp ELISA titres had already been obtained four weeks after priming. The animals received

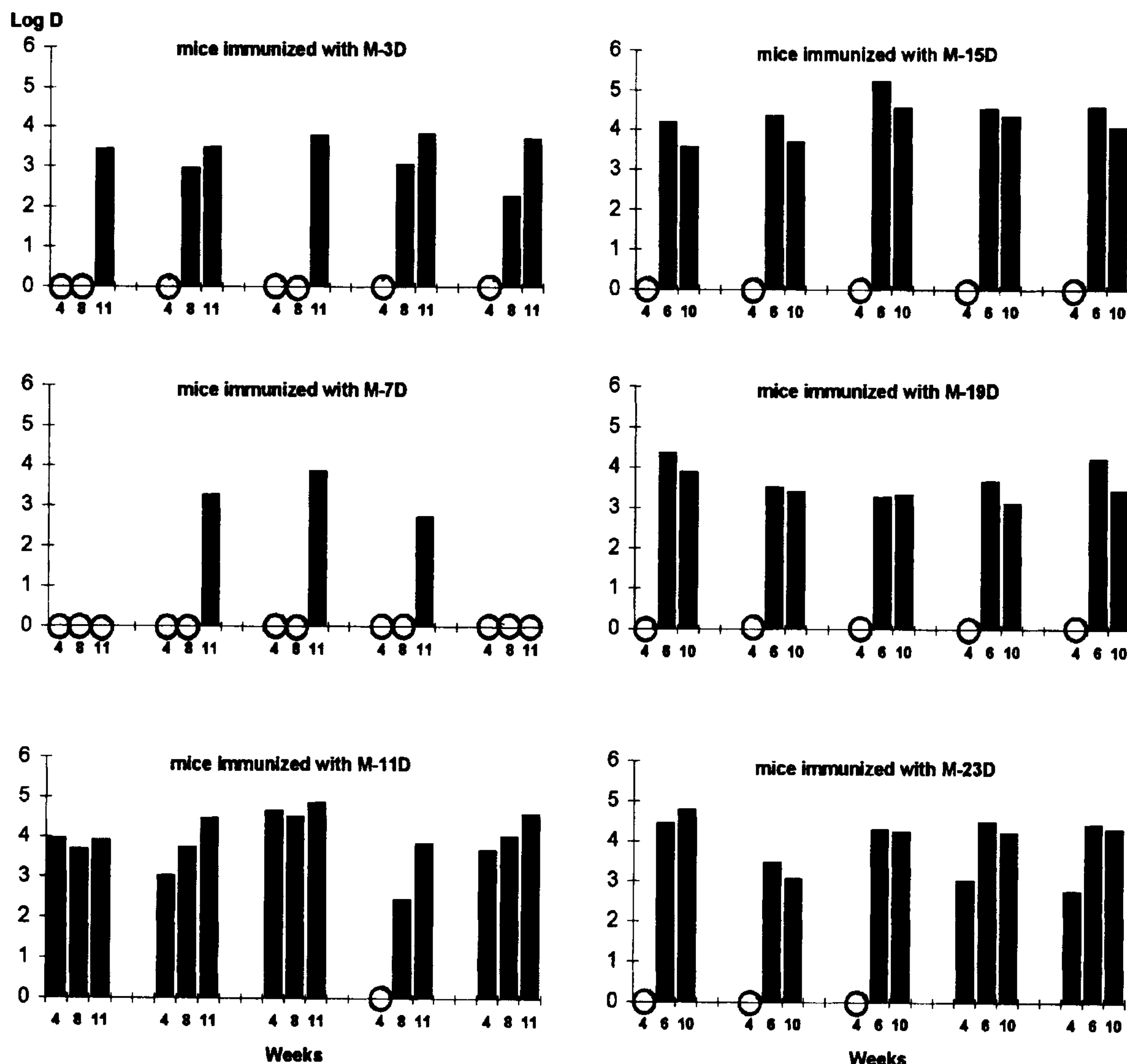


Figure 3 ELISA anti-Dncp titres of mice, after immunization with **M-3D**, **M-7D**, **M-11D**, **M-15D**, **M-19D** and **M-23D**. Each group of columns represents the response of a single animal. The circles mean that the serum titres are ≤ 2.

boosts after five and nine weeks, in the case of anti-**MD-11D** animals after five and seven weeks. It is clear that the C-terminal Dncp obviates all recognition problems of Dncp groups positioned at or near the N-terminal end of melittin peptides.

DISCUSSION

Fundamental studies on peptide immunogenicity may bring parameters into focus which cannot easily be observed with large proteinaceous antigens although they may play their role irrespective of size. A remarkable aspect in our opinion is the earlier finding [2] that rabbit antibody recognizes the N-terminal chain of melittin in the helical form. Melittin is known to assume a helical conformation with two helices bent by 60° relative to each other and separated by Pro of position 14 only in hydrophobic solvents or under conditions mimicking membrane environments whereas in aqueous media it exhibits essentially a random coil [10–12]. It is therefore implied that the helix-specific antibody production could only have been initiated after interaction of the cell-bound recognizing immunoglobulin with the melittin peptide in close contact with the membrane. This obviates the picture of an antigen recognition step which is based solely on B-cell-bound immu-

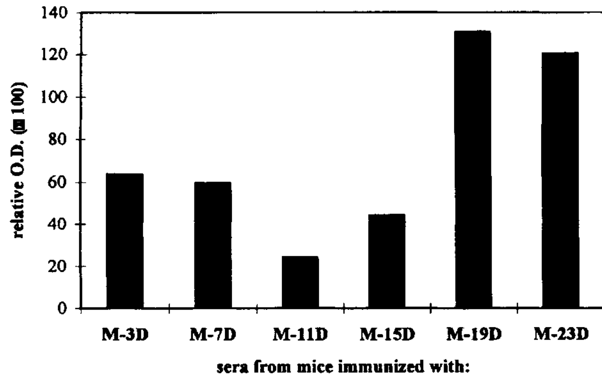


Figure 4 Immunodot assay. Relative O.D. as quantitative measurement for the amount of mainly anti-peptidic IgG responses in mice immunized with **M-3D**, **M-7D**, **M-11D**, **M-15D**, **M-19D** and **M-23D**. The sera were tested against the autologous peptide dotted on nitrocellulose. The bars represent mean values from five antisera collected eight or six weeks after priming (cf. text). The ranges were usually ±20%.

noglobulin sticking out into the aqueous surrounding phase and interacting by its paratope with antigenic epitopes entirely within the aqueous phase.

If we accept the membrane involvement during the recognition step our findings regarding the poor or more precisely late anti-hapten responses with N-terminally monohaptenated melittin peptides readily finds an interpretation. We take it that the Dncp-haptens on the N-terminal α-helix are in rather well-fixed inflexible positions. This is in contrast to the C-terminal Dncp-group which resides on a flexible chain of high polarity which will keep it well within the aqueous layer and thus extended. Recognizing immunoglobulins which can bind Dncp are no doubt abundant. It should be noted in this connection that the parotopes of probably all antibodies are quite large and capable of accommodating up to 22 amino acid residues according to the crystal structure of complexes obtained at medium resolution [13–15]. There must thus exist a considerable number of possibly very diverse antibodies with regard to their overall specificity but with the common feature of strongly binding the small Dncp moiety somewhere within the paratope area. The well-known experience that polyhaptenated Dncp-proteins or in fact most

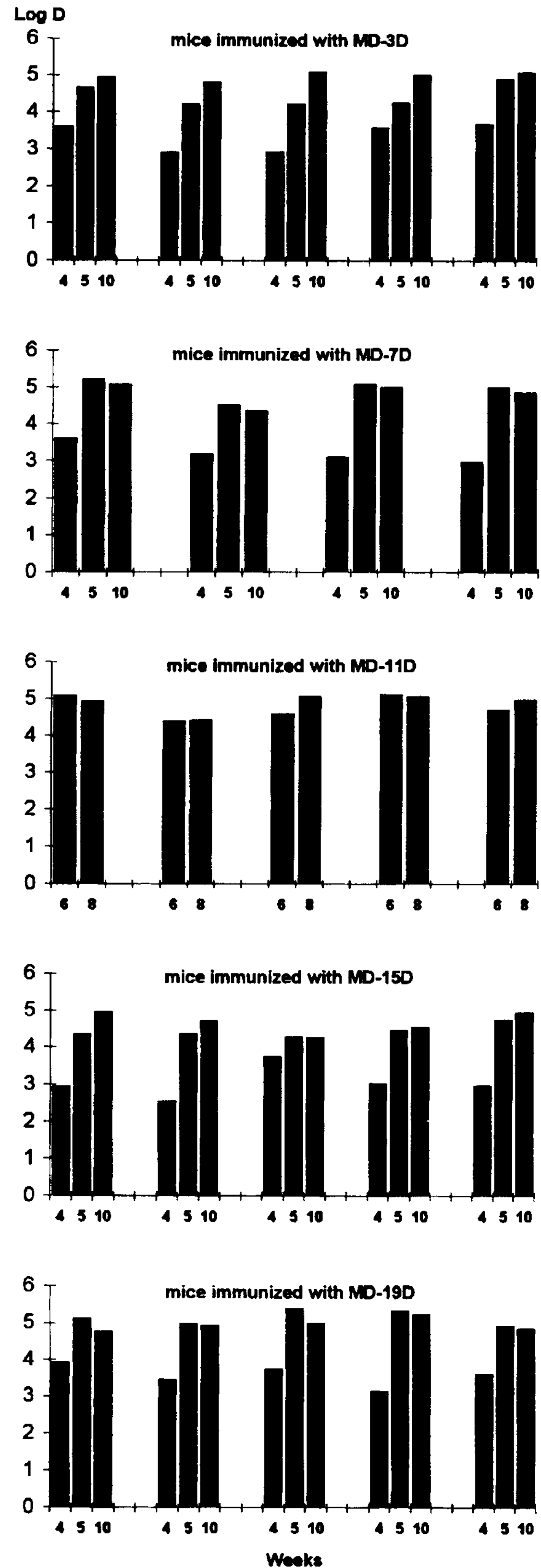


Figure 5 ELISA anti-Dncp titres of mice, after immunization with **MD-3D**, **MD-7D**, **MD-11D**, **MD-15D** and **MD-19D**. Each group of columns represents the response of a single animal.

other haptenated proteins almost never fail to mount an anti- hapten antibody response corroborates this concept. (For a recently found apparent exception see [16].)

Flexibly attached C-terminal Dncp-groups have no difficulty in finding their attachment position within a paratope and thus do not fail to initiate an anti-Dncp antibody response under appropriate conditions as demonstrated earlier [1] as well as in the present context. Helix-attached haptenic groups are configurationally restricted and cannot interact with all potentially capable paratopes. This means that a smaller number of B-cell-bound antibodies with the potential of binding Dncp structures can come into play and start the immunogenic processes. However, at later stages the initial small number of committed B cells becomes unimportant as the clone continues to grow, while clones in other animals that have had a better start, i.e. more committed cells to begin with, have already reached a plateau. We thus explain why N-terminally haptenated peptides (positions 1, 3 and 7) generate late anti-haptenic responses, reaching in due course, however, the level of the more rapid normal responses as shown in Figures 2 and 3.

What remains to be explained is the finding that the impaired responses are less pronounced and less frequently found when the Dncp group is in positions 11, 15, 19 and 23. Of course it can be invoked that the helix towards the C-terminus is shorter and steric restriction therefore less pronounced. Furthermore, positions 11 or 15 are in a region where the helical conformation may be less stable and may be in equilibrium with a considerable proportion of non-fixed structure. It could also be argued that only the N-terminal peptide moiety is firmly in contact with the membrane whereas a considerable portion of the middle section adjacent to the polar C-terminal chain remains more in the aqueous layer, thus keeping some of its flexibility.

The lack of lactoside-specific murine antibody response after immunization with melittin containing N-terminal lactoside was attributed by King *et al.* [6] to the positioning of melittin within the cell membrane. The peptide is assumed to be in a position perpendicular to the lipid bilayer with the C-terminal chain being in contact with the surface and its ionized molecules. The lactoside would thus be buried and inaccessible. While we do not entirely dismiss this notion, we would expect more difficulties in explaining on this basis our late responses (which King *et al.* have not described) in terms of quantitative cellular dynamics.

What is to be emphasized is that King's group as well as our laboratory very definitely assume the involvement of the B-cell membrane during the initiating steps of immunization. This connects the present studies to the work of Schwyzer [17–19] which addresses the problem of optimal interaction between hormone-ligand and its receptor and implies that the target cell membrane will mediate or catalyse these interactions. Membrane interactions were shown to provide a basis for quantitative predictions of pharmacological potencies.

The case of melittin seems somewhat complicated by the fact that it exhibits primary and secondary amphiphilicity [17], i.e. the molecules may assume not only orientations perpendicular to the cell surface as they certainly do when aggregated into pore-forming bundles that also parallel orientations based on secondary amphiphilicity [11, 20]. Since we found rabbit antibodies against the N-terminal melittin helix [2] we might consider a parallel surface position. In order to assess the point definitely we should probably also know the potential and actual distributions of the paratopic areas of the recognizing immunoglobulin within the different layers of the B-cell membrane. Furthermore we should take into account the surface-active, membrane-disrupting properties of melittin [20, 21] although these effects could be slight because comparatively low concentrations of the antigenic peptides are expected during recognition.

From a practical point of view the detection of configurationally restricted interactions between hapten and paratope leading to late responses is of interest since it is conceivable that similar situations may arise with proteinaceous immunogens involving peptidic epitopes. Late responses in vaccination may be valuable and show properties such as direct pathogen neutralization. In epitope analysis weak responses should therefore be followed further by prolonged immunization. This is not common practice at present.

Acknowledgements

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University of Fribourg, and electrospray mass spectrometry at the Institute of Biochemistry, University of Bern, on a Fisons instrument with VG platform.

REFERENCES

1. Z. Zhao, H. Rolli and C. H. Schneider (1995). Immunogenicity of dinitrocarboxyphenylated melittin: The influence of C-terminal chain shortening. N-terminal substitution and prolin insertion at positions 5 and 10. *J. Peptide Sci. 1*, 140–148.
2. R. von Grknigen and C. H. Schneider (1989). Antigenic structure of the hexacosapeptide melittin: Evidence for three determinants, one with a helical conformation. *Immunology 66*, 330–342.
3. T. P. King, L. Kochoumian and A. Joslyn (1984). Melittin-specific monoclonal and polyclonal IgE and IgGl antibodies from mice. *J. Immunol. 133*, 2668–2673.
4. P. F. Fehlner, R. H. Berg, J. P. Tam and T. P. King (1991). Murine T-cell responses to melittin and its analogues. *J. Immunol. 146*, 799–806.
5. T. P. King, M. R. Coscia and L. Kochoumian in: *Molecular Biology and Immunology of Allergens*, D. Kraft and A. Sehon, Eds., p. 11–19, CRC Press, Boca Raton 1993.
6. T. P. King, D. Wade, M. R. Coscia, S. Mitchell, L. Kochoumian and R. B. Merrifield (1994). Structure–immunogenicity relationship of melittin, its transposed analogues and D-melittin. *J. Immunol. 153*, 1124–1131.
7. R. Guenin and C. H. Schneider (1983). Synthesis and anaphylactogenicity of monohaptenic carbohydrate conjugates. *Helv. Chim. Acta 66*, 1101– 1109.
8. M. F. Kasper, C. H. Schneider, H. Rolli, B. Angst and A. L. de Weck (1986). Diagnostic reagents in drug allergy: Immunochemical specificity in the 1,2-diphenyl-pyrazolidinedione series. *Immunobiol. 173*, 98–109.
9. C. S. Hong, B. M. Stadler, M. Waelti and A. L. de Weck (1986). Dot- immunobinding assay with monoclonal anti-IgE antibodies for the detection and quantification of human IgE. *J. Immunol. Meth. 95*, 195–202.
10. C. R. Dawson, A. F. Drake, J. Helliwell and R. C. Hider (1978). The interaction of bee melittin with lipid bilayer membranes. *Biochim. Biophys. Acta 510*, 75–86.
11. H. Vogel and F. Jähnig (1986). The structure of melittin in membranes. *Biophys. J. 50*, 573–582.
12. H. Vogel (1987). Comparison of the conformation and orientation of alamethicin and melittin in lipid membranes. *Biochemistry 26*, 4562– 4572.
13. J. Novotny (1991). *Molec. Immunol. 28*, 201–207.
14. J. Novotny, E. Brucoleri and F. A. Saul (1989). On the attribution of binding energy in antigen-antibody complexes McPC603, D1.3 and HyHEL-5. *Biochemistry 28*, 4735–4749.
15. M. H. V. van Regenmortel (1995). Transcending the structuralist paradigm in immunology affinity and biological activity rather than purely structural considerations should guide the design of synthetic peptide epitopes. *Biomed. Peptides, Proteins Nucl. Acids 1*, 109–116.
16. J. Bondaruk, V. Curcio-Vonlanthen and C. H. Schneider (1995). Basic aspects related to penicillin-allergy skin testing: On the variability of the hapten–paratope interaction. *Allergy 50*, 671–676.
17. R. Schwyzer (1992). Conformations and orientations of amphiphilic peptides induced by artificial lipid membranes: Correlations with biological activity. *Chemtracts - Biochem. Molec. Biol. 3*, 347–379.
18. R. Schwyzer (1991). Peptide–membrane interactions and a new principle in quantitative structure - activity relationships. *Biopolymers 31*, 785–792.
19. R. Schwyzer in: *Peptides 1986*, D. Theodoropoulos, Ed., p. 7–23, de Gruyter, Berlin 1987.
20. T. C. Terwilliger, L. Weissman and D. Eisenberg (1982). The structure of melittin in the form I crystals and its implication for melittin's lytic and surface activities. *Biophys. J. 37*, 353–361.
21. C. E. Dempsey (1990). The actions of melittin on membranes. *Biochim. Biophys. Acta 1031*, 143–161.